Variation in General Esterase Activity within a Population of *Haematobia irritans* (Diptera: Muscidae)

J. H. PRUETT, D. M. KAMMLAH, AND F. D. GUERRERO

Knipling-Bushland U. S. Livestock Insects Research Laboratory USDA–ARS-SPA, Kerrville, TX 78028

J. Econ. Entomol. 94(3): 714–718 (2001)

ABSTRACT Control of the horn fly, *Hematobia irritans* (L.), is generally dependent on chemical insecticides. However, the biology and behavior of the horn fly favors rapid development of insecticide resistance. To prolong the effectiveness of the insecticide option, information is required regarding the mechanisms of insecticide resistance. Metabolic hydrolysis of insecticides by esterases is a detoxification mechanism in many insect species. Measurement of general esterase activity within populations of horn flies may provide a diagnostic tool for resistance management. In this study we evaluated the amount of variation in general esterase activity within female and male horn fly samples from a population that had not been exposed to insecticides for 8 yr. We found considerable variation in general esterase activity within samples of each sex, with females demonstrating the greater variation. The observed variation is thought to be the result of age-structure dynamics within the population. The amount of inherent variation makes it difficult to detect small mean differences between populations, thus limiting the utility of general esterase assays. Thus, effective diagnosis of esterase-mediated resistance mechanisms can only be achieved by the identification of specific detoxification esterases and the design of assays, either biochemical or molecular, for their detection and measurement.

KEY WORDS *Hematobia irritans irritans*, insecticide resistance, esterase

THE HORN FLY, *Hematobia irritans* (L.), is an obligate blood-feeding ectoparasite of cattle responsible for annual economic losses of approximately U.S. $730 million (Drummond et al. 1981, Kunz et al. 1991). Horn fly induced stress and annoyance results in a loss of feed efficiency, reduced rate of gain, and decreased milk production (Campbell 1976, Kinzer et al. 1984). Control of the horn fly has been almost completely dependent upon chemical insecticides (Byford et al. 1999). Unfortunately horn fly biology and behavior favor rapid development of insecticide resistance (Byford et al. 1999). Horn fly mobility ensures treatment of a large percentage of the population, effectively eliminating a refugium of susceptible individuals (Byford et al. 1999).

Mitigation of insecticide resistance within a horn fly population is a complicated task because of the potential interaction of several mechanisms of resistance. A *kdr*- type mutation (*kdr*, knockdown resistance) and a *super kdr*-type mutation have been described in the *para*-type sodium channel gene of the horn fly (Guerrero et al. 1997). These mutations confer a high level of resistance to pyrethroid insecticides (Guerrero et al. 1997). Enzymatic detoxification of pyrethroids by oxidative pathways has also been reported for the horn fly (Sparks et al. 1990). The mixed function oxidase (cytochrome P450) inhibitor, piperonyl butoxide, synergized permethrin toxicity in super-resistant horn flies that also possessed the *kdr* point mutation (Guerrero et al. 1997). In addition, single-fly extract analyses have demonstrated that permethrin susceptible and resistant horn flies possess esterases that can effectively hydrolyze permethrin (Bull et al. 1988, Sparks et al. 1990, Xu and Bull 1995, Pruett et al. 2000).

The effective life span of an insecticide is dependent upon the intensity of the selection pressure for resistance (Devonshire et al. 1998). Options to minimize selection pressure become evident as diagnosis of resistance mechanisms becomes possible (Devonshire et al. 1998). The metabolic esterase, E4 of *Myzus persicae*, has been shown to degrade and sequester insecticidal esters including pyrethroid, carbamate, and organophosphate (OP) insecticides (Devonshire and Moores 1982). Little is known regarding specific metabolic mechanisms of OP resistance in the horn fly. Researchers have attempted to correlate general esterase activity in terms of banding patterns and staining intensity for electromorphs between susceptible and resistant populations but without much diagnostic success (Szalanski et al. 1995, Castiglioni-Ruiz et al. 1997, Guerrero et al. 1999). Microplate assays of single-fly general esterase activity from a collected sample of flies allow the general esterase activity of a horn fly population to be quantified. To statistically compare mean esterase activities from different populations, it is necessary to understand the inherent variability in esterase activity within a horn fly population, particularly a susceptible population. The purpose of the current study is to evaluate the variability in general esterase activity within female and male samples

from a horn fly population that had not been exposed to insecticides for 8 yr.

Materials and Methods

Microplate Esterase Assay. The general esterase activity of single-fly extracts was determined using α-naphthyl acetate as substrate in a microplate technique (Devonshire et al. 1986), as modified by Pruett et al. (2000). Each fly was pulverized on ice in a 1.5-ml conical centrifuge tube containing 100 μl of extraction buffer (20 mM sodium phosphate buffer, pH 7.0). The pulverized fly was centrifuged at 14,000 × *g* in a Hermle centrifuge (National Labnet, Woodbridge, NJ) for 5 min at 4°C. The supernatant fluid was collected and diluted 1:100 with extraction buffer. The diluted sample (50 μl) was placed into the well of a microtiter plate with 150 μl of extraction buffer containing 5.0×10^{-4} M α-naphthyl acetate. The enzyme reaction was allowed to proceed for 30 min at 30°C. At the completion of incubation, 50 μl of extraction buffer containing 0.15% o-dianisidine tetrazotized, and 1.75% sodium dodecyl sulfate was added to each well. The plates were placed in the dark at room temperature, and the color development was allowed to stabilize for 15 min. The resultant optical density (OD) value of each well was measured at 620 nm using a CERES UV900HDi (Biotek, Winooski, VT) plate reader. The experimental OD value for each replicate ($n = 2$) sample was determined by subtracting a plate dye background OD value (buffer + dye) from the resultant OD value, followed by the subtraction of a substrate control OD value (buffer + substrate + dye). Specific activity in terms of μmoles naphthol per minute per fly equivalent was determined from an α-naphthol standard curve ranging from 3.9 to 500 μM.

Collection of Aging Flies from an In Vivo Colony. An in vivo colony of horn flies was established from flies originally collected from cattle located 72.5 km (45 miles) NE of College Station, TX, on the Camp Cooley Ranch during November of 1997. The flies were collected for the purpose of establishing an OP-resistant colony. At the time of collection, flies were bioassayed with the OP diazinon and the LC_{50} concentration was estimated to be 0.029 μg/cm^2 with a resistance ratio of 2.0 (laboratory susceptible strain LC_{50}, 0.015 μg/cm^2) by cloth bioassay (Schmidt et al. 1985). Further laboratory selection with diazinon was not attempted, and within 1 yr the estimated LC_{50} concentration had declined to a susceptible level, at 0.007 μg/cm^2. Thus, flies used in this experiment were not considered resistant to diazinon. A sample of newly emerged, unfed flies was removed from the emergence cage before the release of newly emerged flies onto the host steer. At the appropriate time of host exposure (24, 48, 72, and 144 h), the host steer was swept for fly collection. Collected flies were sexed and stored at −80°C.

Collection of Camp Stanley Horn Flies. At the time of fly collections, the Camp Stanley (San Antonio, TX) horn fly population had not been exposed to insecticides for 8 yr. A cloth bioassay (Schmidt et al. 1985) conducted before fly collection (20 August 1998) estimated the LC_{50} concentration for diazinon to be 0.016 μg/cm^2 for the Camp Stanley population (laboratory susceptible strain LC_{50}, 0.012 μg/cm^2). Flies were collected Monday through Friday mornings for 2 wk by sweep net from heavily infested cattle (>1,000 flies). Collected flies were transported to the Kerrville laboratory, sexed, and frozen at −80°C.

Age-Grading of Female Camp Stanley Horn Flies. Female flies were aged by the age-grading technique for *Stomoxys calcitrans* (L.) as described by Scholl (1980). Age-grading was based upon five stages of gonotrophic development.

Data Analysis. Data were analyzed using SigmaStat software (Kuo et al. 1992). Differences within male and female fly collections relative to esterase activity and age-grading data were analyzed by the Kruskal–Wallis analysis of variance (ANOVA) on ranks, with multiple comparisons made with Dunn's method ($P < 0.05$).

Table 1. Variation in general esterase activity between Camp Stanley female and male horn flies

Day	*n*	Mean ♀ μM/min[a]	SD	*n*	Mean ♂ μM/min	SD
1	100	6.624	3.818	99	4.707	2.278
2	98	6.445	4.544	100	3.611	1.543
3	100	6.787	3.875	100	3.813	1.812
4	87	6.966	4.299	99	3.533	2.157
5	100	6.433	4.278	100	4.232	2.339
8	100	4.687	3.054	100	2.838	1.447
9	100	4.146	2.526	100	4.199	1.600
10	100	6.009	3.854	100	3.717	1.684
11	100	5.750	3.226	100	4.157	1.943
12	100	5.768	3.529	100	4.814	2.447

Fly samples were collected Monday through Friday over a 2-wk period.

[a] μM/min/fly equivalent, fly equivalent = 50 μl of a 1:100 dilution of fly extract.

Results

General Esterase Variation within Samples of Female and Male Horn Flies. Daily measurements of mean general esterase activities for female and male horn flies collected from the Camp Stanley horn fly population are presented in Table 1. Rather large standard deviations within samples suggest considerable variation between individuals, particularly within the female population. Male samples from days 1, 9, and 11 were the only samples that passed a test of normality, and all female samples failed a test of normality. Mean female general esterase activities were lower and more variable on a day-to-day basis in the second week of the study, and a decline in esterase activity was noted on days 8 and 9. This variability resulted in significant differences between observations (Kruskal–Wallis one-way ANOVA on ranks, $H = 59.926$, $df = 9$, $P = < 0.001$). In paired comparisons (Dunn's method, $P < 0.05$), observations made on days 1, 3, 4, and 5 of the study were significantly different from observations made on days 8 and 9 of

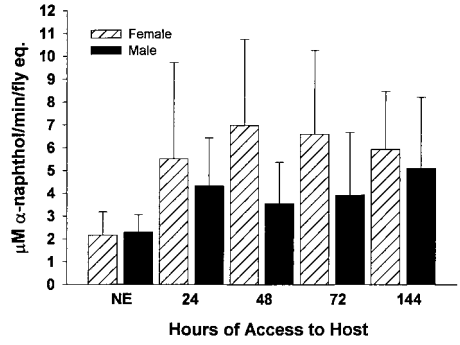

Fig. 1. General esterase activity (mean ± SD) of aging female and male horn flies. Female samples: $n = 20$ for newly emerged (NE), 24, 48, and 72 h, $n = 14$ for 144 h. Male samples: $n = 20$ for NE and 72 h, $n = 9$ for 24 h, $n = 14$ for 48 h and $n = 19$ for 144 h.

Table 2. Mean physiological age and age distribution of Camp Stanley female horn flies collected Monday through Friday over a 2-wk period

Day	Mean	SD	% stage 1[a]	% stage 2	% stage 3	% stage 4	% stage 5
1	3.5	1.080	0.0	10.0	60.0	0.0	30.0
2	2.3	1.160	30.0	30.0	20.0	20.0	0.0
3	2.4	1.897	60.0	0.0	10.0	0.0	30.0
4	2.3	1.059	30.0	20.0	40.0	10.0	0.0
5	2.3	0.949	30.0	10.0	60.0	0.0	0.0
8	3.3	1.252	10.0	10.0	40.0	20.0	20.0
9	2.0	1.633	70.0	0.0	0.0	20.0	10.0
10	1.7	0.949	60.0	10.0	30.0	0.0	0.0
11	2.8	1.398	20.0	30.0	10.0	30.0	10.0
12	1.5	0.707	60.0	30.0	10.0	0.0	0.0

Age-grading based on ovarian development of a sample of $n = 10$.
[a] Stage 1 follicle separated from germarium and spherical, stage 5 egg at maximum size and maturity (Scholl 1980).

the second week. The day 2 general esterase activity differed significantly from day 9, but not day 8. In the second week of the study esterase activity increased toward the end of the week as days 10, 11, and 12 differed significantly from day 9. Male general esterase activity varied less than that of females, although, as with females, a drop in esterase activity was noted on day 8 (2.838 μM/min). Nonetheless, this variation resulted in a significant difference within the male population (Kruskal–Wallis one-way ANOVA on ranks, H = 83.560, df = 9, $P = < 0.001$). This noted variation was almost totally dependent upon the low day 8 esterase activity. In paired comparisons (Dunn's method, $P < 0.05$) days 1, 2, 3, and 5 were significantly different from day 8 of the study. As male esterase activity also increased toward the end of the second week, observations on days 9, 10, 11, and 12 were significantly greater than day 8. In addition, day 1 mean male esterase activity was greater than that of day 4 of week 1 and day 12 of week 2 was greater than days 2 and 4 of week 1.

The Effect of Aging on General Esterase Values. Changes in mean general esterase activity within aging female and male horn flies from an in vivo colony are presented in Fig. 1. Newly emerged flies of both sexes yielded the lowest esterase activity. The mean general esterase activity of newly emerged females was significantly less than for samples obtained at 24 through 144 h of access to the host (Kruskal–Wallis one-way ANOVA on ranks H = 43.798, df = 4, $P = < 0.001$; paired comparison Dunn's method, $P < 0.05$). Mean male general esterase activity of newly emerged flies was significantly lower than for the 24 and 144 h samples (Kruskal–Wallis one-way ANOVA on ranks H = 20.594, df = 4, $P = < 0.001$; paired comparison Dunn's method, $P < 0.05$).

Age-Grading of Female Horn Flies. A subsample ($n = 10$) of Camp Stanley female flies collected daily (Monday through Friday for 2 wk) were age-graded by degree of ovarian development (Table 2). Based on lower mean age-grades it appeared that in the second week of the study the fly population was composed of younger flies. Although a significant difference was found between the observations (Kruskal–Wallis one-way ANOVA on ranks, H = 21.052, df = 9, $P = 0.012$) upon pairwise comparison (Dunn's method $P < 0.05$) only day 1 differed significantly from day 12.

Distribution of General Esterase Activities on Days 1 and 9. Results of general esterase assays of day 1 and day 9 female fly collections ($n = 100$ for both) were pooled into six activity groupings and presented in a histogram (Fig. 2). These data clearly reflect a shift in distribution for day 9 toward the lower activity groupings, particularly the lowest grouping (0.00–2.99 μM/min). On day 1, 17 of the females collected were in the lowest grouping and on day 9, 41 females yielded general esterase activities that placed them in the lowest grouping, representing an increase of 2.4 times.

Discussion

Metabolic carboxylesterases are implicated in the detoxification and sequestration of OP insecticides in the mosquito (Karunaraine et al. 1998, Callaghan et al. 1998), the sheep blowfly (Hughes and Devonshire 1982; Campbell et al. 1997, 1998), and the peach potato

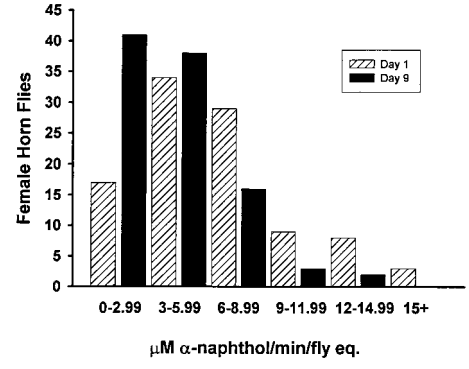

Fig. 2. Histogram of the distribution of day 1 and day 9 female general esterase activities. General esterase activities sorted into six activity groupings (0–2.99, 3–5.99, 6–8.99, 9–11.99, 12–14.99, 15+).

aphid (Field and Devonshire 1998). While studies of horn fly OP hydrolyzing esterases are lacking, horn flies are known to possess esterase(s) that hydrolyze the pyrethroid insecticide permethrin (Bull et al. 1988, Sparks et al. 1990, Xu and Bull 1995, Pruett et al. 2000). Relative to OP-susceptible flies, OP-resistant horn flies have been found to possess elevated esterase activity (Guerrero et al. 1999). Thus, pyrethroid and OP hydrolyzing esterases of the horn fly, although poorly defined at present may have an important role in resistance to either OP or pyrethroid insecticides, and an elevated level of general esterase activity may be a diagnostic indicator of insecticide resistance particularly with respect to OPs.

An important management tool for monitoring developing insecticide resistance would be a diagnostic test for esterase-mediated resistance mechanisms. Qualitative and quantitative measurement of general esterase activity with α-naphthyl acetate as substrate has revealed elevated esterase activity within OP-resistant horn flies (Guerrero et al. 1999). This observation suggests that measurement of general esterase activity could find utility as a diagnostic tool in the management of insecticide use, and prolong the period of time before development of insecticide resistance within the target species. However, before development of such an assay, it is imperative to quantify variability in general esterase activity within an OP-susceptible population. In the current study we found considerable variation in general esterase activity within a population of diazinon-susceptible horn flies. The observed variability would make it difficult to accurately diagnose subtle differences between populations of flies. The female population demonstrated a higher degree of general esterase variation than did the males. The noted variation is most probably the result of physiological variability within the age-structure of the population. The data presented in this study and previously (Pruett et al. 2000) support the hypothesis that general esterase activity increases with aging, and that newly emerged flies have a much lower level of general esterase activity than do more mature flies. Thus, an increase in the proportion of newly emerged flies within the population would lower the mean general esterase activity. The data that we present for age-graded female flies generally supports the hypothesis that during the second week of the study the fly population was composed of younger flies (Table 2). This difference was mostly reflected in a shift of the percentage of flies from later stages of development to earlier stages of development. Considering the variation within the sample, a larger sample size may have provided more support for this conclusion. However, more convincing evidence for a shift in the age-structure of the population is presented in Fig. 2, a histogram of general esterase activity distribution within samples of days 1 and 9 female flies. On day 1, 17 of 100 females had general esterase values within the lowest grouping, whereas on day 9 the number had increased to 41 of 100 females in the lowest grouping. Considering that esterase activity increases with aging, particularly after 24 h, the increase in the number of females with lower esterase activities on day 9 is concluded to represent a change in the age-structure of the sample.

These data suggest that a simple measurement of general esterase activity is limited as a diagnostic tool because of natural variability in general esterase activity within the fly population. As a result of this inherent variability in general esterase activity only large differences in means can be reliably detected. Also it is known that substantial resistance as a function of metabolic detoxification may be reflected by a subtle, if any, change in general esterase activity (ffrench-Constant and Devonshire 1988). Therefore, the development of reliable diagnostic tools awaits the identification of specific enzymes involved in metabolic detoxification, and the design of immunochemical, biochemical, or molecular assays of specific esterase activity. Information obtained from specific assays will provide more meaningful information for use in mitigating the development of insecticide resistance.

Acknowledgments

The authors thank Brenda K. Burkett and Melinda May for their excellent technical assistance, Matt Waldon for horn fly rearing and colony maintenance, and Dennis Ingenheutt for the field collection of horn flies.

References Cited

Bull, D. L., R. L. Harris, and N. W. Pryor. 1988. The contribution of metabolism to pyrethroid and DDT resistance in the horn fly (Diptera: Muscidae). J. Econ. Entomol. 81: 449–458.

Byford, R. L., M. E. Craig, S. M. DeRouen, M. D. Kimball, D. G. Morrison, W. E. Wyatt, and L. D. Foil. 1999. Influence of permethrin, diazinon and ivermectin treatments on insecticide resistance in the horn fly (Diptera: Muscidae). Int. J. Parasitol. 29: 125–135.

Callaghan, A., T. Guillemaud, N. Makate, and M. Raymond. 1998. Polymorphisms and fluctuations in copy number of amplified esterase genes in *Culex pipiens* mosquitoes. Insect Mol. Biol. 7: 295–300.

Campbell, J. B. 1976. Effect of horn fly control on cows as expressed by increased weaning weights of calves. J. Econ. Entomol. 69: 711–712.

Campbell, P. M., J. F. Trott, C. Claudianos, K. A. Smyth, R. J. Russell, and J. G. Oakeshott. 1997. Biochemistry of esterases associated with organophosphate resistance in *Lucilia cuprina* with comparisons to putative orthologues in other Diptera. Biochem. Genetics 35(1/2): 17–40.

Campbell, P. M., R. D. Newcomb, R. J. Russell, and J. G. Oakeshott. 1998. Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, *Lucilia cuprina.* Insect Biochem. Mol. Biol. 28: 139–150.

Castiglioni-Ruiz, L., E. M. Hermione, B. de Campos, and C. R. Ceron. 1997. Esterase patterns in four Brazilian populations of *Haematobia irritans.* Cytobios 90: 81–94.

Devonshire, A. L., and G. D. Moores. 1982. A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach-potato aphids *(Myzus persicae).* Pestic. Biochem. Physiol. 18: 235–246.

Devonshire, A. L., G. D. Moores, and R. H. ffrench-Constant. 1986. Detection of insecticide resistance by immunological estimation of carboxylesterase activity in *Myzus persicae* (Sulzer) and cross reaction of the antiserum with *Phorodon humuli* (Schrank) (Hemiptera: Aphididae). Bull. Entomol. Res. 76: 97–107.

Devonshire, A. L., L. M. Field, S. P. Foster, G. D. Moores, M. S. Williamson, and R. L. Blackman. 1998. The evolution of insecticide resistance in the peach-potato aphid, *Myzus persicae.* Philos. Trans. R. Soc. Lond. B. 353: 1677–1684.

Drummond, R. O., G. Lambert, H. E. Smalley, and C. E. Terrill. 1981. Estimated losses of livestock to pests, pp. 111–127. *In* D. Pimentel [ed.], CRC handbook of pest management in agriculture, vol. 1. CRC Boca Raton, FL

ffrench-Constant, R. H., and A. L. Devonshire. 1988. Monitoring frequencies of insecticide resistance in *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) in England during 1985–1986 by immunoassay. Bull. Entomol. Res. 78: 163–171.

Field, L. M., and A. L. Devonshire. 1998. Evidence that the E4 and FE4 esterase genes responsible for insecticide resistance in the aphid *Myzus persicae* (Sulzer) are part of a gene family. Biochem. J. 330: 169–173.

Guerrero, F. D., R. C. Jamroz, D. Kammlah, and S. E. Kunz. 1997. Toxicological and molecular characterization of pyrethroid-resistant horn flies, *Haematobia irritans:* Identification of *kdr* and *super-kdr* point mutations. Insect Biochem. Mol. Biol. 27: 745–755.

Guerrero, F. D., J. H. Pruett, S. E. Kunz, and D. M. Kammlah. 1999. Esterase profiles of diazinon-susceptible and -resistant horn flies (Diptera: Muscidae). J. Econ. Entomol. 92: 286–292.

Hughes, P. B., and A. L. Devonshire. 1982. The biochemical basis of resistance to organophosphorus insecticides in the sheep blowfly, *Lucilia cuprina.* Pestic. Biochem. Physiol. 18: 289–297.

Karunaraine, S.H.P.P., A. Vaughan, M. G. Paton, and J. Hemingway. 1998. Amplification of a serine esterase gene is involved in insecticide resistance in Sri Lankan *Culex tritaeniorhynchus.* Insect Mol. Biol. 7(4): 307–316.

Kinzer, H. G., W. E. Houghton, J. M. Reeves, S. E. Kunz, J. D. Wallace, and N. S. Urquhart. 1984. Influence of horn flies (*Haematobia irritans*) on weight loss in cattle with notes on prevention of loss by insecticide treatment. Southwest. Entomol. 9: 212–217.

Kunz, S. E., K. D. Murrell, G. Lambert, L. F. James, and C. E. Terril. 1991. Estimated losses of livestock to pests, pp. 69–98. *In* D. Pimentel [ed.], CRC handbook of pest management in agriculture, vol. 1, 2nd ed. CRC, Boca Raton, FL.

Kuo, J., E. Fox, and S. McDonald. 1992. SigmaStat statistical software for working scientists. Jandel Scientific, San Rafael, CA.

Pruett, J. H., D. D. Oehler, D. M. Kammlah, and F. D. Guerrero. 2000. Evaluation of horn flies (Diptera: Muscidae) from a pyrethroid susceptible colony for general and permethrin esterase activities. J. Econ. Entomol. 93: 920–924.

Schmidt, C. D., S. E. Kunz, D. Petersen, and J. L. Robertson. 1985. Resistance of horn flies (Diptera: Muscidae) to permethrin and fenvalerate. J. Econ. Entomol. 78: 402–406.

Scholl, P. J. 1980. A technique for physiologically age-grading female stable flies, *Stomoxys calcitrans* (L.). Research bulletin 298. Institute of Agriculture and Natural Resources, University of Nebraska-Lincoln.

Sparks, T. C., R. L. Byford, M. E. Craig, B. L. Crosby, and C. Mckenzie. 1990. Permethrin metabolism in pyrethroid-resistant adults of the horn fly (Diptera: Muscidae). J. Econ. Entomol. 83: 662–665.

Szalanski, A. L., W. C. Black IV, and A. B. Broce. 1995. Esterase staining activity in pyrethroid resistant horn flies (Diptera: Muscidae). J. Kans. Entomol. Soc. 68: 303–312.

Xu, G., and D. L. Bull. 1995. Biochemical properties of esterases in pyrethroid-resistant and -susceptible strains of the horn fly (Diptera: Muscidae). J. Econ. Entomol. 88: 1186–1191.

Received for publication 26 September 2000; accepted 14 February 2001.